

## Evidence for McKusick's hypothesis of deficient collagen cross-linking in patients with homocystinuria

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### Abstract

Osteoporosis occurs commonly in homocystinuria. The underlying pathobiochemical mechanism remains unclear: disturbed cross-linking of collagen has been suggested but this hypothesis has not been fully tested, nor have studies on collagen synthesis been performed. We therefore used recently available noninvasive tests for collagen synthesis and cross-linking to examine 10 patients with homocystinuria. Synthesis of collagen type I and type III was not different from age-matched healthy controls as reflected by comparable plasma levels of carboxyterminal propeptide of type I procollagen (PICP) and of plasma levels of N-terminal propeptide of procollagen type III (PIIINP). Collagen type I cross-links expressed by serum carboxyterminal telopeptide of collagen type I (ICTP) were  $1.14 \pm 0.24 \mu\text{g/l}$  in the patient group versus  $3.29 \pm 0.32 \mu\text{g/l}$  in the control group. This significant reduction of cross-links in the group with homocystinuria did not correlate with serum homocysteine or homocysteic acid concentrations. Our data clearly indicate that the disturbed cross-linking hypothesis still holds and that the bone manifestations of homocystinuria are not due to deficient collagen synthesis.

**Keywords:** Procollagen peptide; Collagen metabolism; Homocystinuria; Homocysteine; Homocysteic acid; Osteoporosis; Osteopenia

### 1. Introduction

Patients with homocystinuria develop osteoporosis, subluxation of the ocular lens, mental retardation and vascular disease; the overall risk of these patients developing osteoporosis has been reported to be 50% by age 16 [1]. McKusick first proposed that excess homocysteine might interfere with the normal synthesis of collagen cross-links [2] and some indirect evidence for this hypothesis was provided by the finding of increased acid solubility of collagen from skin biopsies in two out of four patients with homocystinuria [3]. Homocystinuric patients presenting with decreased precursor aldehydes of skin collagen have been described [4].

A mechanism similar to that reported for D-penicillamine, which shows structural analogy with homocysteine, has also been proposed. D-penicillamine decreases collagen solubility by blocking lysyl- or hydroxylysyl aldehyde residues which are known cross-link precursors [5]. Inhibi-

tion of the formation of Schiff base bifunctional cross-links has been suggested as well [6]. In vitro studies have shown that homocysteine inhibits cross-linking and fibril formation of isolated collagen in solution although in relatively high concentrations [4]. This is not a specific finding as it is well known that other amino acids can inhibit fibril formation of collagen at relatively low concentrations; an example is the reaction of the amino groups of arginine with the carbonyl/aldehydic residues [7]. Although high concentrations of homocysteine have been reported to inhibit lysyl oxidase, such concentrations are unlikely to occur in vivo [8].

The hypotheses mentioned above and in a recent review [9] require confirmation. No in vitro or in vivo studies investigating other mechanisms have been published so far and, in particular, no work has been reported examining collagen synthesis in vivo or in vitro. We therefore studied collagen type I synthesis by measuring plasma carboxyterminal propeptide of type I procollagen (PICP), cross-linked carboxyterminal telopeptide of collagen type I (ICTP) and the synthesis of collagen type III as represented by the

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N-terminal propeptide of procollagen type III (PIIINP) in plasma.

We found normal synthetic parameters for collagens type I and III but a significant decrease of cross-linked carboxyterminal telopeptide of collagen type I in patients with homocystinuria.

## 2. Patients and methods

### 2.1. Patients

Plasma of 10 patients with hyperhomocysteinemia were obtained from a group of patients treated at the University Hospital Nijmegen. Nine of them had cystathionine beta synthase deficiency and one had the thermolabile form of methylenetetrahydrofolate reductase deficiency [10]. Cystathionine beta synthase deficiency was established by severely elevated levels of homocystein and methionine and decreased (<5% of normals) enzyme activity in fibroblasts. The clinical characterization is given in Table 1.

EDTA blood was drawn and centrifuged 10 min at  $3000 \times g$  within 30 min and stored at  $-20^{\circ}\text{C}$ .

Plasma of 20 age-matched healthy subjects served as controls.

### 2.2. Methods

#### 2.2.1. Determination of plasma total homocysteine and homocysteic acid levels

For the HPLC determination the principle of Fermo with modifications to allow the concomitant detection of homocysteic acid was followed [11]. Briefly, 100  $\mu\text{l}$  of distilled water (HPLC grade Merck, Germany) and 100  $\mu\text{l}$  of 9 M urea were added to 100  $\mu\text{l}$  of plasma. 10  $\mu\text{l}$  of 2

$\mu\text{M}$  cysteic acid (Sigma) in 0.1 M HCl were added as internal standard, followed by the addition of 0.1 ml of 3 M NaBH<sub>4</sub> in 0.1 M NaOH mixed with DMSO (2:1 v/v). To prevent foaming during the reduction step 30  $\mu\text{l}$  of *n*-amylalcohol were used. After a 30-min incubation period at  $50^{\circ}\text{C}$  and protein precipitation with 500  $\mu\text{l}$  of 6% perchloric acid, 500  $\mu\text{l}$  of the supernatant were mixed with 100  $\mu\text{l}$  of freshly prepared iodoacetic acid (0.1 M in 9.22 M borate buffer). After the addition of 100  $\mu\text{l}$  of 3 M NaOH 200  $\mu\text{l}$  of the obtained solution were derivatized with 100  $\mu\text{l}$  of 400  $\mu\text{M}$  o-phthalaldehyde solution as described elsewhere [12].

100- $\mu\text{l}$  aliquots were injected into the port of the HPLC system consisting of the pump system Applied Biosystems model 400 and a Spectroflow 980 fluorescence detector. The wavelengths used were 230 nm for excitation and 417 nm for emission. The Shimadzu C-R6A Chromatopac integrator was used for data handling.

A C18 Kromasil 100 analytical (5  $\mu\text{m}$ ) column ( $250 \times 4.6$  mm) was connected to a second column of that kind with the dimensions of  $150 \times 4.6$  mm. The mobile phase consisted of solvent A (water/sodium propionate buffer/acetonitrile = 60:30:10) and solvent B (water/acetonitrile/methanol = 45:30:25). The flow rate was 1.3 ml/min during 24 min, then 1.0 ml for 6 min, 1.3 ml during 22 min and 1.4 ml/min during 10 min. Standards used in the experiments were homocysteine hydrochloride, homocysteic acid and cysteic acid, all from Sigma.

#### 2.2.2. Determination of plasma parameters for collagen metabolism and cross-linking

Commercially available radioimmunoassays for plasma carboxyterminal propeptide I (PICP, Orion Diagnostica), cross-linked carboxyterminal telopeptide of collagen type I (ICTP, Orion Diagnostica) and N-terminal propeptide collagen type III (PIIINP, Orion Diagnostica) were applied

Table 1  
Clinical characterization of patients

| Patient No.        | Age                       |          | Sex | Height <sup>b</sup> (cm) | Weight <sup>b</sup> (kg) | Marfanoid <sup>c</sup> | Treatment |        |         |         |
|--------------------|---------------------------|----------|-----|--------------------------|--------------------------|------------------------|-----------|--------|---------|---------|
|                    | At diagnosis <sup>a</sup> | At study |     |                          |                          |                        | Vit B6    | Folate | Vit B12 | Betaine |
| 1 E53              | 25                        | 31       | m   | 188                      | 80                       | —                      | +         |        |         |         |
| 2 M61              | 23                        | 33       | m   | 190                      | 89                       | +                      | +         |        | +       |         |
| 3 B61              | 26                        | 33       | m   | 191                      | 77                       | +                      | +         | +      |         | +       |
| 4 B58              | 27                        | 36       | m   | 191                      | 87                       | +                      | +         | +      | +       | +       |
| 5 S25              | 61                        | 65       | f   | 165 <sup>d</sup>         | 56                       | +                      |           |        |         |         |
| 6 V43 <sup>e</sup> | 50                        | 50       | m   | 170                      | 84                       | —                      | +         | +      |         |         |
| 7 D67              | 3                         | 27       | m   | 187                      | 77                       | —                      | +         | +      |         | +       |
| 8 D66              | 4                         | 28       | m   | 184                      | 84                       | —                      | +         | +      |         | +       |
| 9 U78              | 7                         | 16       | f   | 171                      | 64                       | —                      | +         | +      | +       |         |
| 10 C62             | 31                        | 31       | f   | 180                      | 63                       | +                      |           |        |         |         |

<sup>a</sup> Treatment started since diagnosis except in patient 6.

<sup>b</sup> Height/weight at study.

<sup>c</sup> Marfanoid because of dolichostenomelia, arachnodactyly or thorax deformation.

<sup>d</sup> Height in the presence of kyphoscoliosis.

<sup>e</sup> 5-Methylenetetrahydrofolate reductase deficiency because of thermolability.

and the supplier's instructions were followed.

### 2.2.3. Statistical calculations

The Mann-Whitney *U*-test was used for the comparison of groups and linear regression analysis for the correlation of data [13]. Statistical significance was accepted at the  $P < 0.05$  level.

## 3. Results

The results are listed in Tables 2 and 3.

Plasma homocysteine and homocysteic acid were significantly higher in the group with homocystinuria. There was no correlation between homocyst(e)ine and homocysteic acid.

The collagen synthesis parameters for type I and type III collagen did not differ between controls and patients. ICTP levels, the plasma parameter for collagen type I

Table 2  
Results of plasma total homocysteine homocysteic acid and the plasma parameters for collagen metabolism and cross-linking

| Patient No. | Homocyst(e)ine $\mu\text{M}/\text{l}$ | Homocysteic acid $\mu\text{M}/\text{l}$ | PICP <sup>a</sup> $\mu\text{g}/\text{l}$ | PIIINP <sup>b</sup> $\mu\text{g}/\text{l}$ | ICTP <sup>c</sup> $\mu\text{g}/\text{l}$ |
|-------------|---------------------------------------|---|--|--|--|
| 1 E53       | 53                                    | 8                                       | 146                                      | 4.4  | 1.3                                      |
| 2 M61       | 41                                    | 9                                       | 152                                      | 4.2  | -  |
| 3 B61       | 101                                   | 9                                       | 130                                      | 2.9  | -  |
| 4 B58       | 79                                    | 8                                       | 176                                      | 4.1  | 1.4                                      |
| 5 S25       | 238                                   | 9                                       | 184                                      | 5.1  | 1.2                                      |
| 6 V43       | 108                                   | 6                                       | 122                                      | 3.3  | -  |
| 7 D67       | 90                                    | 8                                       | 166                                      | 2.9  | -  |
| 8 D66       | 199                                   | 10                                      | 173                                      | 4  | 0.8                                      |
| 9 V78       | 106                                   | 10                                      | 142                                      | 3.3  | -  |
| 10 C62      | 252                                   | 9                                       | 162                                      | 4.2  | 1  |
| Controls    |                                       |   |  |  |  |
| 1           | 16                                    | 2                                       | 144                                      | 4.2  | 3.2                                      |
| 2           | 14                                    | 1                                       | 162                                      | 4.1  | 3.4                                      |
| 3           | 10                                    | 1                                       | 125                                      | 4.3  | 3.1                                      |
| 4           | 12                                    | 2                                       | 136                                      | 2.6  | 4  |
| 5           | 16                                    |   | 146                                      | 2.7  | 3.1                                      |
| 6           | 13                                    | 1                                       | 156                                      | 3.1  | 3.2                                      |
| 7           | 18                                    | 2                                       | 170                                      | 3.2  | 3.3                                      |
| 8           | 7                                     | 1                                       | 172                                      | 4  | 3.2                                      |
| 9           | 10                                    | 2                                       | 180                                      | 4  | 3.4                                      |
| 10          | 11                                    | 1                                       | 173                                      | 4.1  | 3.5                                      |
| 11          | 11                                    | 2                                       | 142                                      | 3.4  | 3.6                                      |
| 12          | 12                                    | 3                                       | 160                                      | 5.1  | 3.3                                      |
| 13          | 14                                    | 1                                       | 136                                      | 4.2  | 3.2                                      |
| 14          | 16                                    | 2                                       | 142                                      | 3.3  | 3  |
| 15          | 15                                    | 1                                       | 129                                      | 3.4  | 4.1                                      |
| 16          | 15                                    |   | 162                                      | 3.6  | 2.9                                      |
| 17          | 16                                    |   | 186                                      | 3.2  | 3  |
| 18          | 12                                    | 2                                       | 170                                      | 4.1  | 3.1                                      |
| 19          | 8                                     | 1                                       | 164                                      | 4  | 2.9                                      |
| 20          | 10                                    | 1                                       | 141                                      | 4.1  | 3.3                                      |

<sup>a</sup> PICP reflecting collagen type I formation (carboxyterminal propeptide of procollagen type I).

<sup>b</sup> PIIINP reflecting collagen type III formation (N-terminal propeptide of procollagen type III).

<sup>c</sup> ICTP reflecting collagen type I cross-links (cross-linked carboxyterminal telopeptide of collagen type I) ↓ undetectably low.

Table 3

Means and S.D. of parameters evaluated in the study

|          |       | HC $\mu\text{M}/\text{l}$ | HCA $\mu\text{M}/\text{l}$ | PICP $\mu\text{g}/\text{l}$ | PIIINP $\mu\text{g}/\text{l}$ | ICTP $\mu\text{g}/\text{l}$ |
|----------|-------|---------------------------|----------------------------|-----------------------------|-------------------------------|-----------------------------|
| Patients | Means | 126.7                     | 8.6                        | 155.3                       | 3.84                          | 1.14                        |
|          | S.D.  | 75.39                     | 1.17                       | 20.41                       | 0.72                          | 0.24                        |
| Controls | Means | 12.8                      | 1.53                       | 154.8                       | 3.735                         | 3.29                        |
|          | S.D.  | 2.98                      | 0.62                       | 17.6                        | 0.612                         | 0.32                        |

cross-linking, however, were significantly lower in the patient group and undetectably low in half the plasmas examined.

There was no correlation between homocyst(e)ine, homocysteic acid and any parameter of collagen metabolism and cross-linking.

## 4. Discussion

In this study, we have demonstrated normal parameters for collagen type I and III synthesis but decreased collagen cross-linking.

PICP is nearly an ideal analyte of collagen formation, as it is formed and set free in a stoichiometric relationship to the amount of type I collagen synthesized and deposited in the tissue. Furthermore, the antigen measured in serum is homogenous and of the same size as the authentic propeptide. Its elimination from serum takes place via mannose receptor-mediated endocytosis mainly by the endothelial cells of the liver [14]. PICP is a reliable indicator for collagen synthesis, for example in bone formation; good examples are the increase of PICP in bone forming metastasis of prostate cancer [15], the decrease of PICP in osteoporotic women [16], the decrease of PICP in osteogenesis imperfecta [17] or the decrease following systemic glucocorticoid therapy [18]. In our study no difference between plasma PICP of patients with homocystinuria and healthy probands was observed, although both compounds, homocysteine and homocysteic acid are toxic in higher concentrations and thus could have led to inhibition of bone collagen synthesis. PIIINP reflecting procollagen type III synthesis and studied as a minor collagen in bone but major collagen in skin, was unaffected as well.

ICTP is formed during the maturation of collagen fibres and representing type I collagen specific cross-links. This cross-linked carboxyterminal telopeptide of collagen type I is cleared by the kidneys. Collagen type I degradation results in increased serum levels of ICTP correlating with calcium kinetics [19] and bone histomorphometric indices [20]; good examples are the increased ICTP in breast cancer with bone-degrading metastasis, rheumatoid arthritis with osteolysis [21] or multiple myeloma [22]. In our study a significant reduction of plasma ICTP was noted in the homocystinuric subjects. However, no correlation between homocysteine or homocysteic acid levels and plasma ICTP was found. It is possible that, since homocysteine

levels were much higher in the cases than controls, a threshold effect is operating rather than a dose–response effect. Another possibility is that tissue rather than plasma homocysteine might correlate better with plasma ICTP.

The interpretation of these results on ICTP can be therefore only speculative. Based on the suggested mechanism leading to osteoporosis in homocystinuria reduction of ICTP must be interpreted as measuring a collagen split product with reduced cross-linking, thus fully compatible with mechanisms listed in the introduction. The sulfhydryl residue of homocysteine is able to block reactive carbonyls representing precursors of collagen cross-links [5]. It is therefore not surprising that serum cross-linked carboxyterminal telopeptide of type I collagen is reduced in our homocystinuric patients and supports the hypothesis of deficient cross-linking in homocystinuria as first proposed by McKusick [2].

Reduction and subsequent cleaving of disulfide cross-links of bone collagen by homocysteine would be an alternative mechanism for the development of osteoporosis but is not relevant as collagen type I does not contain significant cystein residues required for disulfide cross-linking [23].

ICTP is considered a parameter either for collagen cross-linking or degradation; we found its reduction and interpret the reduction as representing decreased collagen cross-links and not decreased degradation of collagen which would not fit into the disease, even if a cytotoxic effect of homocysteine was taken into consideration [24].

Our results show that reduced synthesis of collagens is not responsible for osteoporosis in homocystinuria and we provide data indicating reduced collagen cross-linking. We now propose studying further parameters of impaired cross-linking as, e.g., the determination of urinary pyridinoline cross-links which in turn would strengthen the cross-linking hypothesis.

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